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Localization and Fine Mapping of Antigenic Sites on the Nucleocapsid Protein N of Porcine Reproductive and Respiratory Syndrome Virus with Monoclonal Antibodies

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The purpose of this study was to analyze the antigenic structure of the nucleocapsid protein N of the Lelystad virus isolate of porcine reproductive and respiratory syndrome virus (PRRSV) and to identify antigenic differences between this prototype European isolate and other North American isolates. To do this, we generated a panel of monoclonal antibodies (mAbs) directed against the N protein of Lelystad virus and tested them in competition assays with other N-specific mAbs described previously (Drew *et al.,* 1995; Nelson *et al.,* 1993; van Nieuwstadt *et al.,* 1996). Four different competition groups of mAbs were identified. Pepscan analysis with solid-phase dodecapeptides was used to identify specific antigenic regions in the N protein that were bound by the mAbs. In this pepscan analysis, we found that the mAb of the first competition group reacted with linear peptides whose core sequences consisted of amino acids 2–12 (site A), the mAbs of the second group reacted with peptides whose core sequences consisted of amino acids 25–30 (site B), and the mAb of the third group reacted with peptides whose core sequences consisted of amino acids 40–46 (site C). However, the fourth group of mAbs binding to an antigenic region, provisionally designated as domain D, reacted very weakly or did not react at all with solid-phase dodecapeptides.To further characterize the structure of the epitopes in domain D, we produced chimeric constructs composed of the N protein sequences of Lelystad virus and another arterivirus lactate dehydrogenase-elevating virus, which was used because its N protein has similarity in amino acid sequence and hydropathicity profile but does not react with our mAbs. When the mAbs specific to domain D were tested for binding to the chimeric N proteins expressed by Semliki Forest virus, we found that the regions between amino acids 51–67 and amino acids 80–90 are involved in the formation or are part of the epitopes in domain D. Therefore, we conclude that the N protein contains four distinct antigenic regions. The epitopes mapped to sites A–C are linear, whereas the epitopes mapped to domain D are more conformation dependent or discontinuous. Sites A and C contain epitopes that are conserved in European but not in North American isolates; site B contains epitopes that are conserved in European and North American isolates; and site D contains epitopes that are either conserved or not conserved in European and North American isolates. The antigenic regions identified here might be important for the development of diagnostic test for PRRSV in particular tests that discriminate between different antigenic types of PRRSV.

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) belongs to the family of arteriviruses together with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (Meulenberg *et al.,* 1993; Plagemann and Moennig, 1992). It causes abortions in pregnant sows and respiratory distress in piglets (Wensvoort *et al.,* 1991; Collins *et al.,* 1992), and it causes huge economic losses worldwide. Infection most frequently occurs by the respiratory route, and virus replication in the respiratory tract is followed by viremia and dissemination throughout the body. The genome of PRRSV is 15 kb in length and contains genes encoding the RNA-dependent RNA polymerase (ORF1a and ORF1b) and genes encoding structural proteins (ORFs 2–7; Meulenberg *et al.,* 1993). ORF5 encodes the major envelope glycoprotein, designated GP_5 (Mardassi *et al.,* 1996; Meulenberg *et al.,* 1995b). The ORFs 2–4 encode glycoproteins designated GP_2, GP_3, and GP_4, respectively. These glycoproteins are less abundantly present in purified virions of the Lelystad virus (LV) isolate of PRRSV (Meulenberg *et al.,* 1996; Van Nieuwstadt *et al.,* 1996). The GP_5 protein forms a disulfide-linked heterodimer with the membrane protein M encoded by ORF6 (Mardassi *et al.,* 1996). The nucleocapsid protein N is encoded by ORF7 (Mardassi *et al.,* 1996; Meulenberg *et al.,* 1996). The analysis of the genome sequence of PRRSV isolates from Europe and North America and their reactivity with monoclonal antibodies (mAbs) has proved that they represent two different antigenic types. The isolates from these continents are genetically distinct and must have diverged from a common ancestor relatively long ago (Kapur *et al.,* 1997; Murtaugh *et al.,* 1995).

Many studies have shown that the N protein is the most antigenic protein of PRRSV. For example, pigs infected with PRRSV develop strong antibody responses against the N

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TABLE 1

Properties of mAbs that Bind to the N Protein and Were Used in This Study

mAb	Isotype	Titer in IPMA[a] with LV	Reaction with US isolates[b]	Binding to antigenic region	Pepscan on N protein of LV[c]
138.22	IgG1	25	−	A	1-MAGKNQSQKKKKS-13
125.1	IgG1	250	+	B	19-NGQPVNQLCQLLGAMIKS-36
126.9	IgG2b	250	+	B	19-NGQPVNQLCQLLGAMIKSQ-37
NS95	IgG1	NA[d]	+	B	19-NGQPVNQLCQLLGAMIKS-36
NS99	IgG1	NA[d]	+	B	19-NGQPVNQLCQLLGAMIKSQ-37
126.15	IgG1	250	−	C	35-KSQRQQPRGGQAKKKKP-51
122.17	IgG1	2000	+	D	±[e]
130.2	IgG1	250	+	D	±[e]
130.4	IgG1	250	+	D	±[e]
131.7	IgG1	250	+	D	±[e]
131.9	IgG1	250	+	D	±[e]
SDOW17	IgG1	250	+	D	±[e]
WBE1	IgG2a	1000	−	D	±[e]
WBE4	IgG1	1000	−	D	±[e]
WBE5	IgG1	2000	−	D	−
WBE6	IgG1	1000	−	D	−

[a] Reciprocal of the highest dilution of HRPO-conjugated mAb (4 mg IgG/ml) positive in an indirect immunoperoxidase monolayer assay of lung alveolar macrophages infected with LV. Similar titers were obtained using other European isolates of PRRSV.

[b] Reactivity in IPMA with 10 U.S. isolates from different states, which were characterized previously (Kapur *et al.,* 1997).

[c] The core amino acid sequence of the peptides, reacting with the mAbs is indicated.

[d] NA = not available.

[e] ± indicates that binding of the mAb was observed but was batch dependent or did not fulfill the criterion for being above background (see Results).

protein (Loumba *et al.,* 1996; Meulenberg *et al.,* 1995a; Sanz *et al.,* 1995). Furthermore, most mAbs produced from mice that were immunized with (partially) purified virions of PRRSV were directed against the N protein (Drew *et al.,* 1995; Nelson *et al.,* 1993; Rodriquez *et al.,* 1997; van Nieuwstadt *et al.,* 1996). Therefore, it was suggested to be a useful antigen for the development of routine diagnostic tests to detect serum antibodies against PRRSV (Denac *et al.,* 1997; Meulenberg *et al.,* 1995a; Rodriquez *et al.,* 1997). For the design of these tests, it is important to know the antigenic structure of the N protein, in particular the similarities and differences between European and North American isolates of PRRSV. The ability to differentiate between these isolates is becoming critical because vaccine virus of the North American antigenic type has recently been introduced into Europe.

Thus although the antigenic importance of the N protein is widely recognized, little has been known until now about the antigenic structure of the N protein or to what extent the N proteins of the North American and European isolates differ antigenically. The purpose of this study was to identify the antigenic regions of the N protein using a variety of mAbs from various laboratories. Some of the mAbs recognized only the N protein of European isolates, whereas others recognized the N protein of both European and North American isolates. We used the mAbs in competition assays to analyze whether they bound similar or distinct antigenic regions on the N protein. Pepscan analysis was used to more precisely map the binding sites of the mAbs. The binding sites of the mAbs that did not react in pepscan analysis were further characterized in studies with chimeric N proteins. With these methods, four antigenic regions were identified: three antigenic sites contained linear epitopes, and one antigenic domain contained conformation-dependent or discontinuous epitopes.

RESULTS

Production and specificity of mAbs recognizing the N protein

In a previous report, we described the isolation of two mAbs (122.17 and 126.9) that reacted with the N protein of LV and other European and North American isolates of PRRSV (van Nieuwstadt *et al.,* 1996). Besides these two mAbs, seven other N protein-specific mAbs were developed (Table 2). mAbs 125.1, 130.2, 130.4, 131.7, and 131.9 recognized epitopes on the N protein that are conserved in both European and North American isolates of PRRSV, whereas mAbs 138.22 and 126.15 recognized an epitope that is conserved in European isolates but not in North American isolates (Table 2). The whole panel of N-specific mAbs reacted with the N protein of LV in the immunoperoxidase monolayer assay (IPMA), immunoprecipitation assay, Western blots, and ELISA and did not neutralize the virus.

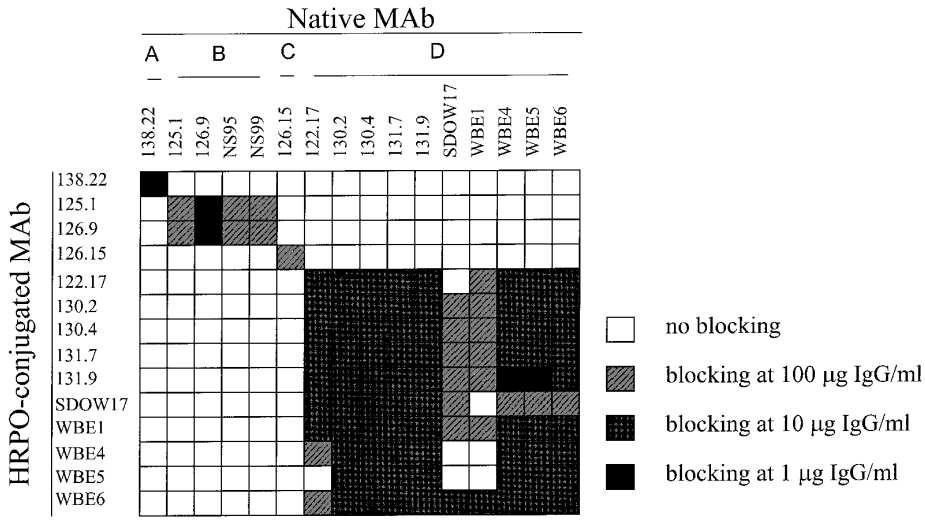

FIG. 1. Epitope mapping by competitive binding in IPMA. Macrophages were seeded onto M96 wells and infected with LV. At 24 h after infection, cells were fixed and incubated with the nonconjugated and HRPO-conjugated mAbs as described under Materials and Methods. The highest dilution of the nonconjugated mAb completely blocking the binding of the conjugated mAb is indicated. The four distinct antigenic regions (A–D) bound specifically by the mAbs are indicated.

Identification of four competition groups of mAbs

The ability of the mAbs to block the binding of another mAb that was conjugated with horseradish peroxidase (HRPO) was assessed in IPMA with LV-infected macrophages. Besides the nine mAbs isolated in our laboratory, four N-specific mAbs raised against a U.K. isolate of PRRSV (WBE1, WBE4, WBE5, and WBE6; Drew *et al.,* 1995), one N-specific mAb raised against U.S. isolate VR2332 (SDOW17; Nelson *et al.,* 1993) and two N-specific mAbs raised against the LV isolate (NS95 and NS99; Nelson *et al.,* 1996) were tested. Four distinct antigenic sites were identified (Fig. 1). HRPO-conjugated mAbs 138.22 and 126.15 were blocked only by their homologous native mAbs, suggesting that they recognize two distinct epitopes at sites that were designated A and C, respectively. Sites A and C are specific for the N protein of European PRRSV isolates because mAbs 138.22 and 126.15 do not recognize North American PRRSV isolates (Table 2). HRPO-conjugated mAbs 126.9 and 125.1 were blocked by their homologous native mAbs and reciprocally by each other, indicating that they bind to adjacent or overlapping epitopes located at a distinct site, designated B. mAbs NS95 and NS99, which were available only in their native form, also blocked the binding of mAbs 126.9 and 125.1, indicating that they bind to site B as well. Site B is probably conserved in European and North American PRRSV isolates because mAbs 126.9, 125.1, NS95, and NS99 recognize PRRSV isolates from both continents.

Interestingly, the fourth and largest group of mAbs that competed mainly reciprocally with each other for binding of the N protein consisted of mAbs that recognize both European and North American isolates of PRRSV (122.17, 130.2, 130.4, 131.7, 131.9, and SDOW17) and mAbs that recognize European isolates only (WBE1, WBE4, WBE5, and WBE6). Within this group, nonreciprocal blocking was observed (Fig. 1). Whereas reciprocal competition of mAbs indicates that their epitopes overlap or are adjacent on the antigen, nonreciprocal competition may be explained in several ways. The affinity of the two mAbs may differ greatly; the binding of one mAb may induce a change in conformation that alters the binding of the other mAb; the binding of one mAb may sterically hinder the binding of the other mAb; or conjugating one mAb to peroxidase may alter the ability of the mAb to bind. Therefore, the information gained from nonreciprocal

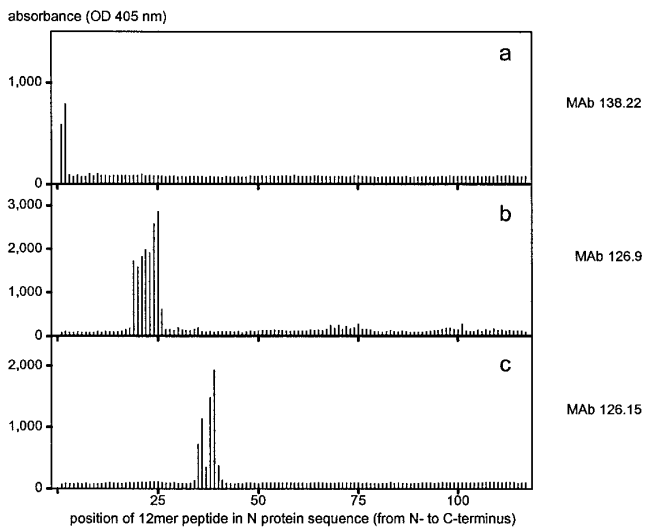

FIG. 2. Pepscan analysis of mAbs on overlapping 12-mer peptides covering residues 1–128 of the N protein. The scans of mAbs 138.22, 126.9, and 126.15 are shown in a, b, and c, respectively. These scans are representative for the mAbs directed to sites A–C, respectively, as discussed under Results and shown in Table 2.

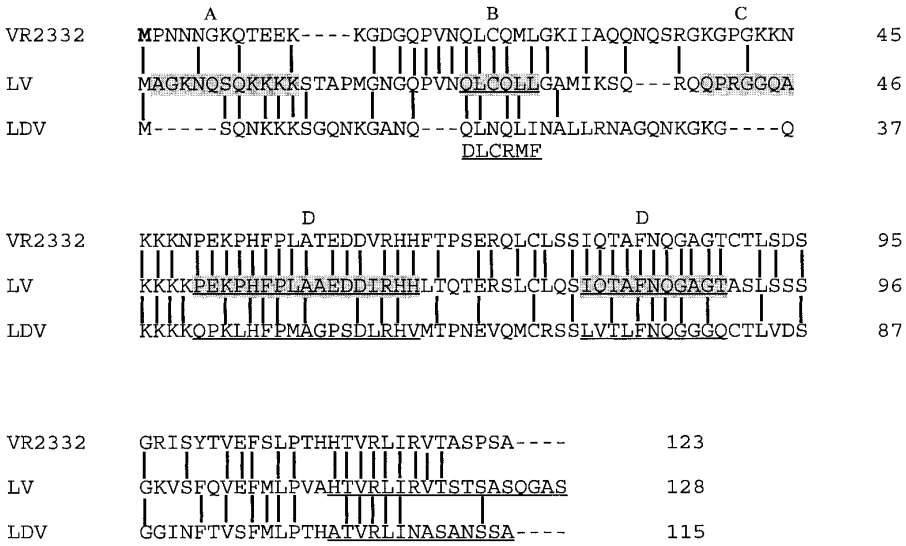

FIG. 3. Location of antigenic binding sites in the N protein sequence of LV and comparison of this N protein sequence with those of North American strain VR2332 and LDV. Sites A–C and domain D are shown in shadow. The amino acid sequences of LV that were substituted for the corresponding amino acid sequences of LDV to map domain D are underlined. The amino acids of the N protein of EAV that were inserted between amino acids 25–30 to mutate site B are shown underlined below the LDV sequence. Identical amino acids are connected with vertical bars.

competition is not straightforward. Nevertheless, the data suggest that the epitopes of mAbs 122.17, 130.2, 130.4, 131.7, 131.9, WBE4, WBE5, and WBE6 are adjacent or partially overlapping within one antigenic domain, provisionally designated D. Even though the picture for SDOW17 and WBE1 was less clear, their binding sites also seem to be in close proximity to domain D.

Fine mapping of three linear antigenic sites in the N protein by pepscan analysis

When the fine specificity of the mAbs was further investigated with synthetic peptides (12 mers) from the N protein in pepscan analysis, 6 of the 16 mAbs reacted positively (Fig. 2, Table 2). mAb 138.22 reacted to two consecutive overlapping peptides covering amino acids 1–13, with the sequence AGKNQSQKKKK (site A) as core. mAbs 125.1, 126.9, NS95, and NS99 reacted with peptides covering amino acids 19–37, with the sequence QLCQLL as core (site B). mAb 126.15 reacted with peptides covering amino acids 35–51, with the sequence QPRGGQA as core (site C). The three distinct linear binding sites identified for these mAbs are in agreement with the results of blocking experiments described above and with their reactivity with North American isolates. The amino acid sequence of sites A and C defined by mAbs 138.22 and 126.15, which bind only European isolates of PRRSV, is very different in the N protein of VR2332 (Fig. 3) and other North American isolates (Kapur *et al.,*1997). In contrast, the amino acid sequence of site B, defined by mAbs 125.1, 126.9, NS95, and NS99, which bind both European and North American isolates, is conserved in the N protein sequence of PRRSV isolates from both continents (Fig. 3).

The mAbs specific for domain D could not be mapped with certainty due to too low reactivity above background to be regarded as antigenic or a variable reactivity between batches, or both. mAbs 130.2, 130.4, 131.7, 131.9, WBE1, and WBE5 reacted very weakly with peptides covering amino acids 50–74, whereas mAb SDOW17 reacted very weakly with peptides covering amino acids 20–37 (data not shown). Because of the good reactivity in other immunochemical tests with recombinant protein and virus (Tables 2 and 3), the epitope of each of these mAbs is probably discontinuous or dependent on the conformation of the N protein. mAbs 122.17 and 130.2 exhibited low affinity to more than one stretch of amino acids between residues 19–36 and 50–74 (data not shown). Binding of mAbs to more than one stretch of amino acids in pepscan has been observed before and is additional evidence that the epitope is discontinuous (Posthumus *et al.,* 1990; Korth *et al.,* 1997).

Analysis of the epitopes in domain D using chimeric N proteins

In our first attempt to map domain D, various deletions were made at the 3′ end of ORF7, resulting in truncated N proteins that lacked amino acids at the C terminus. These truncated N proteins were expressed by Semliki Forest virus in BHK-21 cells and stained in IPMA with the N-specific mAbs. However, the N protein with the smallest deletion of 31 N-terminal amino acids was not stained by the D-specific mAbs and was only very weakly stained by the mAbs mapped to sites A–C. Thus the C-terminus of the N protein appeared to be important for its conformation, stability, or both (data not shown).

TABLE 2

Staining of Chimeric N Proteins Expressed by Semliki Forest Virus in BHK-21 Cells in IPMA

		Staining with mAbs in IPMA			
Plasmid	Mutation	138.22 A	125.1/126.9/NS95/NS99 B	126.15 C	122.17/130.2/130.4/131.7/131.9/SDOW17/WBE1/WBE4/WBE5/WBE6 D
pABV470	–	+++	+++	++	+++
pABV462	25–30	+++	–	++	+++
pABV518	51–67	++	++	+	–
pABV460	80–90	++	++	+	–
pABV471	111–124	+++	+++	++	+++

Consequently, we further mapped domain D with constructs of ORF7 expressing chimeric N proteins, thereby keeping the conformation of the N protein as native as possible. Because the D-specific mAbs produced in our laboratory recognized both European and North American isolates of PRRSV, the regions that were most conserved between the N protein of LV and the North American prototype VR2332 were mutated. The nucleotide sequence coding for amino acids 51–67, 80–90, and 111–128 was substituted for a sequence that codes for the corresponding amino acids of LDV (Fig. 3). For completion, site B (amino acids 25–30), which is also conserved in European and North American isolates, was mutated. Because the amino acid sequence of the N protein of LV was very similar to that of the N protein of LDV in site B, this region was substituted for a region encoding the corresponding amino acids of the N protein of EAV (Fig. 3). When the mutated and wild-type N proteins were expressed in BHK-21 cells using the Semliki Forest virus expression system and were tested with the N-specific mAbs in IPMA, the D-specific mAbs reacted identically (Table 3). Their binding was disrupted by mutations between amino acids 51–67 and 80–90 but not by mutations between amino acids 111–128 or amino acids 25–30 (site B). As expected, the N proteins with LDV sequences between amino acids 51–67 and 80–90 were still stained by mAbs directed against sites A–C. However, fewer cells were stained, and the brightness of the staining was less than that observed for the wild-type N protein and the N proteins mutated in amino acids 25–30 (site B) or amino acids 111–128 (Fig. 4). This was most likely caused by a lower expression of the N proteins containing mutations between amino acids 51–67 or 80–90 because a lower yield of these mutant N proteins compared with the other N proteins was also obtained when equal amounts of transcripts were translated *in vitro* (data not shown). As expected, the N protein that contained EAV sequences in site B was not recognized by mAbs mapped to site B (by pepscan analysis) but was still recognized by mAbs that mapped to sites A or C or domain D. These data indicate that amino acids 51–67 and 80–90 are participating to form the conformation-dependent or discontinuous epitopes mapped to domain D.

DISCUSSION

In this work, we identified four distinct antigenic regions in the N protein of PRRSV. Three sites, designated A–C, contain linear epitopes, and these were mapped between amino acids 2–12, 25–30, and 40–46, respectively. However, the fourth region, designated domain D, contains conformation-dependent or discontinuous epitopes that are (partially) composed of amino acids 51–67 and 80–90. Interestingly, not only mAbs produced against the LV isolate of PRRSV (122.17, 130.2, 130.4, 131.7, and 131.9) but also mAbs produced against the H2 U.K. isolate (WBE1, WBE4, WBE5, and WBE6) and against the North American isolate VR2332 (SDOW17) bound to domain D. It is remarkable that immunization of mice with LV resulted in the production of D-specific mAbs that recognized both European and North American isolates, whereas immunization of mice with the closely related H2 U.K. isolate resulted in D-specific mAbs that recognized European strains only, except for WBE1, which recognized certain Canadian isolates (Le Gall *et al.,* 1997). We do not have an explanation for these results. Within the group of mAbs specific for domain D, some inconsistent reactions were observed in competitive binding assays using SDOW17 and WBE1. Drew *et al*. (1995) concluded that WBE1, SDOW17, and WBE4–6 probably bind to different regions in the N protein. A distinct antigenic site for SDOW17 is in line with the observation that SDOW17 does recognize North American isolates of PRRSV, whereas WBE1, WBE4, WBE5, and WBE6 do not. However, in our study, when we mutated two conserved regions of the N protein (amino acids 51–67 and 80–90), not only was the binding of mAbs 122.17, 130.2, 130.4, 131.7, 131.9, and SDOW17 that recognize the N protein of European and North American isolates disrupted, but also the binding of mAbs WBE1, WBE4, WBE5, and WBE6 that recognize only European isolates was disrupted. Apparently, the introduction of mutations between amino acids 51–67 and 80–90 also induces changes in the epitopes recognized by WBE1, WBE4, WBE5, and WBE6 that may consist (partially) of nonconserved amino acids present at positions 60 and 64 and other nonconserved residues in flanking regions

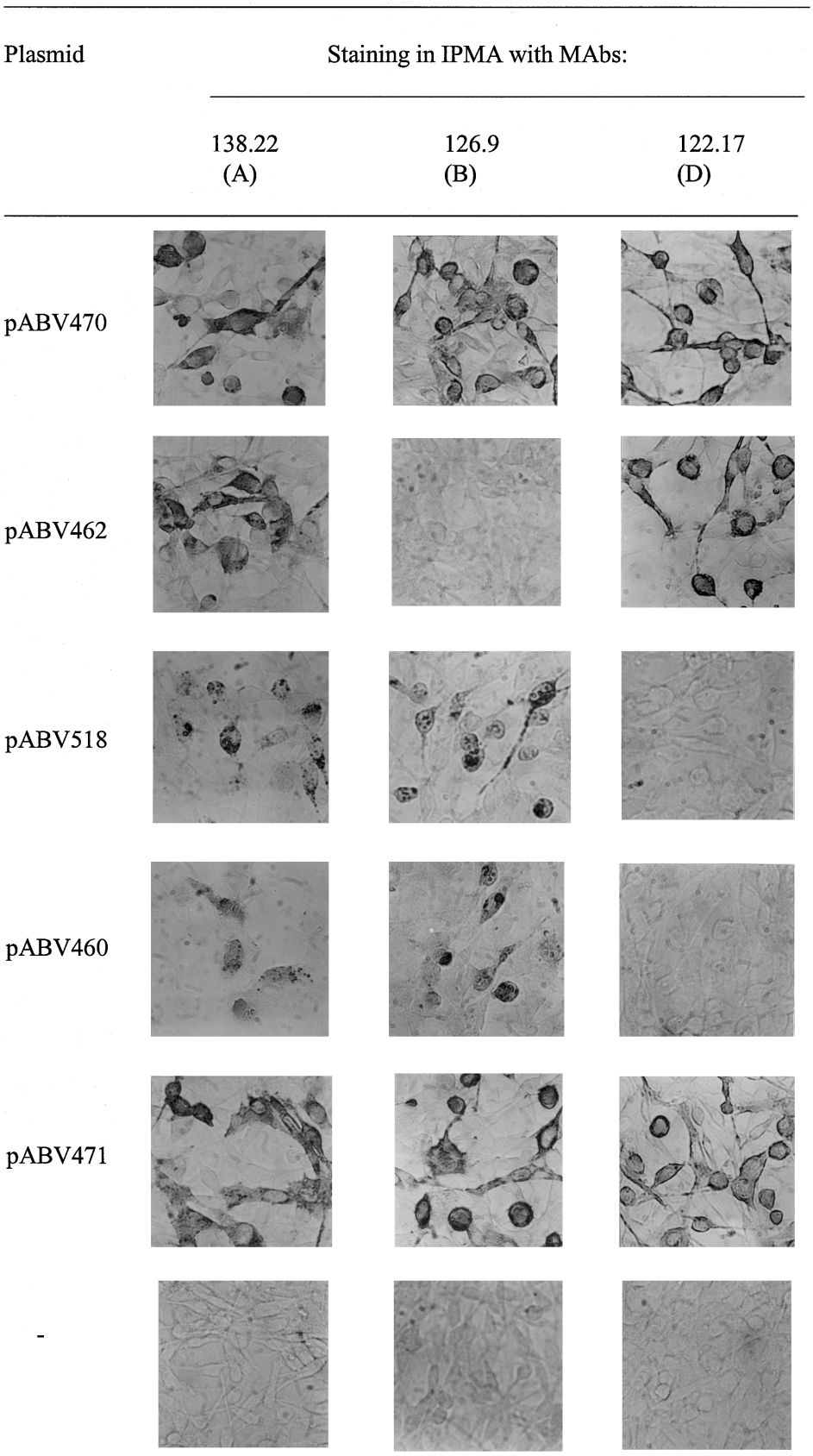

FIG. 4. Immunoperoxidase staining of BHK-21 cells transfected with RNAs from recombinant SFV constructs expressing chimeric and wild-type N proteins. BHK-21 cells were transfected with transcripts from pABV470, pAB462, pABV518, pABV460, and pABV471 and mock transfected (−). At 24 h after tranfection, cells were stained with mAbs 138.22 (site A), 126.9 (site B), and 122.17 (domain D) in IPMA.

TABLE 3

Sequence of Primers Used in PCR to Clone Wild-Type and Chimeric ORF7 Genes

Name	Sequence[a]	Incorporated restriction site
LV98	5′-CCAGCAA*CCT*AGGGG*AGG*ACAGGCCAAAAAGAAAAAGCAGCCGAAGCTACATTTTCCCATGGCTGGTCCATCTGAC-3′	*Eco*NI
LV99	5′-CGTCTGG*ATCGAT*TGCAAGCAGAGGGAGCGTTCAGTCTGGGTGAGGACGTGCCGGAGGTCAGATGGACCAGCC-3′	*Cla*I
LV101	5′-GCTTGCA*GGCGCCC*CGTGACGCTTTTCAATCAAGGCGGAGGACAGGCGTCGCTTTCATCCA-3′	*Nar*I
LV102	5′-ATGT*CCCGGGC*TAAGCGGCGGAGGAATTAGCAGAAGCGTTAATCAGGCGCTGTGTAGCAGCAACCGGCAG-3′	*Sma*I
LV108	5′-GGAGTG*GTTAAC*CTCGTCAAGTATGGCCGGTAAAAACCAGAGCC-3′	*Hpa*I
LV112	5′-CCATTCACCTGACTGT*TTAATTAA*CTTGCACCCTGA-3′	*Pac*I
LV134	5′-TGGGGAA*TGGCCA*GCCAGTCAATGACCTGTGCCGGATGTTTGGTGCAATGATAAAGTCC-3′	*Msc*I

[a] Nucleotides in italics indicate the restriction sites. The mutations with respect to the LV ORF7 sequence are underlined.

(Fig. 3). The fact that a C-terminal deletion of 31 amino acids completely destroyed the binding of all D-specific mAbs (data not shown) further confirmed that the conformation of the N protein is important for the binding of these mAbs. Further mutagenesis of individual residues between amino acids 51–90 is needed to determine which residues are essential for the specific binding of the different mAbs to the N protein. Recently, Nelson *et al*. (1997) described a vaccine strain PrimePac PRRS virus that had lost its reactivity with mAb SDOW17 during serial passage in cell culture. This loss was caused by a single amino acid change at position 61 (Asp to Tyr) in domain D, a finding that agrees with our results. Furthermore, Le Gall *et al*. (1997) showed that mAb WBE1 recognized certain Canadian strains and therefore sequence analysis of the ORF7 gene of these isolates might determine which amino acids are important for binding of WBE1.

Because the majority of mAbs raised against different PRRSV strains mapped to domain D, this region seems to be an immunodominant region, at least in mice. Rodriquez *et al*. (1997) also found that three of the seven mAbs produced against a Spanish isolate of PRRSV were directed against domain D of the N protein. However, in contrast to the mAbs investigated here, their mAbs were recognizing a linear peptide consisting of amino acids 50–66.

Preliminary results have indicated that porcine sera containing antibodies directed against both European and North American PRRSV isolates can interfere with the binding of D-specific mAbs in a blocking ELISA or complex-trapping binding ELISA in which a recombinant N protein product is used (Meulenberg *et al.,* 1995a, unpublished results). Therefore, the D-specific mAbs and perhaps also the B-specific mAbs directed at conserved regions of the N protein are valuable for developing a diagnostic test to detect serum antibodies against PRRSV isolates in pigs worldwide. We are currently investigating whether mAbs that bind to sites A and C might also be used for the development of a diagnostic test discriminating between European and North American isolates or vaccine strains based on these antigenic types. This might be successful because sequence analysis (Kapur *et al.,* 1996; Suarez *et al.,* 1996) and typing of different isolates with our A- and C-specific mAbs (de Kluyver *et al.,* 1995; current study) have shown that sites A and C are conserved in European isolates but different in North American isolates. In addition, site B and domain D are interesting regions to mutate in the recently established infectious clone of LV (Meulenberg *et al.,* 1998) and to create viruses that can be serologically differentiated from field virus and therefore may be promising mutants for marker vaccine development against PRRSV.

MATERIALS AND METHODS

Cells and viruses

The European prototype Ter Huurne strain of LV was isolated in 1991 (Wensvoort *et al.,* 1991). The North American prototype VR2332 strain was isolated in 1992 by Collins *et al*. (1992). LV and VR2332 were grown on CL2621 cells as described previously (Van Nieuwstadt *et al.,* 1996). Macrophages were maintained as described previously (Wensvoort *et al.,* 1991). BHK-21 cells were maintained in Glasgow minimal essential medium (GIBCO BRL/Life Technologies Ltd.), according to the method of Liljeström and Garoff (1993).

Antisera

The production and characterization of the mAbs 122.17 and 126.9 directed against the N protein of LV have been described by van Nieuwstadt *et al*. (1996). In addition to these, seven other N-specific mAbs were generated from mice immunized with purified LV virions (van Nieuwstadt *et al.,* 1996) and are listed in Table 1. The hybridomas producing these mAbs were derived from six consecutive fusion experiments: (I) mAb 122.17, (II) mAb 125.1, (III) mAbs 126.9 and 126.15, (IV) mAbs 130.2 and 130.4, (V) mAbs 131.7 and 131.9, and (VI) mAb 138.22. mAbs WBE1, WBE4, WBE5, and WBE6, which recognize the N protein of European but not that of North American

isolates of PRRSV (Drew *et al.,* 1995) were kindly provided by T. Drew (New Haw, U.K.). mAbs SDOW17, NS95, and NS99 recognize the N protein of both European and North American isolates of PRRSV (Nelson *et al.,* 1993, 1996) and were a gift of D. Benfield (Brookings, U.S.A.).

mAb competition

The N-specific monoclonal antibodies were purified from the culture medium of the hybridoma cells using Protein G–Sepharose 4 Fast Flow (Pharmacia) according to the protocol of the manufacturer. The purified IgG was conjugated to HRPO using a modification of the method of Wilson and Nakane (1978). The conjugated mAbs were diluted to a final concentration of 4 mg/ml. Competitive assays were made using these reagents in (IPMA) to detect blocking of the binding of the HRPO-conjugated mAbs by homologous and heterologous native mAbs. Macrophages were infected in M96 plates with LV at an m.o.i. of 0.1. At 24 h after infection, plates were fixed as described previously (Wensvoort *et al.,* 1986) and were incubated with the native mAb at concentrations of 100, 10, and 1 μg/ml in 0.5 M NaCl, 0.05% Tween 80, and 4% horse serum for 1 h at 37°C. Then, the conjugated mAb was added at concentrations that gave optimal staining: 2 μg/ml (mAb 122.17, WBE5), 4 μg/ml (mAbs WBE1, WBE4, and WBE6), or 16 μg/ml (mAbs 125.1, 126.9, 126.15, 130.2, 130.4, 131.7, 131.9, 138.22, SDOW17) in 0.5 M NaCl, 0.05% Tween 80, and 4% horse serum. After incubation for 1 h at 37°C, cells were washed with 0.15 M NaCl and 0.5% Tween 80 and stained with 3-amino-9-ethyl-carbazole, as described by Wensvoort *et al*. (1986).

Epitope mapping by pepscan

A complete set of solid-phase overlapping dodecapeptides was synthesized covering the amino acid sequence of the N protein of LV (128 residues; Meulenberg *et al.,* 1993). The synthesis of solid-phase peptides on polyethylene rods and immunoscreening with an ELISA type of analysis were carried out according to established pepscan procedures (Geysen *et al.,* 1984). The monoclonal antibodies were tested at various concentrations containing 0.3–200 μg of IgG/ml. Peptides were considered to represent antigenic sites if individual absorbance signals of two or more neighboring peptides reproducibly reached a value of more than twice the background. Background is defined as the average absorbance value of 20 consecutive low signals with a variation coefficient of $\leq$20%.

Plasmid constructions

Two oligonucleotides located upstream (LV108) and downstream (LV112) of ORF7 were used to amplify and clone the ORF7 gene in pGEM-T, resulting in pABV431. The sequences and positions of these and other oligonucleotides used to amplify fragments of ORF7 are listed in Table 1. In addition, four different chimeric constructs were made by PCR-directed mutagenesis. The sequences coding for amino acids 25 and 28–30 (site B; Fig. 3) were substituted for the corresponding sequences of the EAV N protein. This was accomplished by PCR amplification of ORF7 with LV134 and LV112. The mutated DNA fragment was introduced in pABV431 using the *Msc*I and *Pac*I site, which resulted in pABV455. The region of ORF7 encoding amino acids 51–67 was substituted for the corresponding region of LDV ORF7. pABV431 was digested with *EcoN*I and *Cla*I and ligated to a PCR fragment produced with primers LV98 and LV99 digested with the same enzymes. This plasmid was designated pABV463. The region of ORF7 encoding amino acids 80–90 was substituted for the corresponding region of the LDV ORF7 gene. The ORF7 gene of LV was mutated in a PCR with primers LV101 and LV112. The obtained fragment was digested with *Nar*I and *Pac*I and ligated to pABV431 digested with *Cla*I and *Pac*I. This resulted in pABV453. Finally, the region encoding the C-terminal part of the N protein (amino acids 111–128) was replaced for a sequence encoding the corresponding amino acids of the N protein of LDV. The ORF7 gene was amplified with primers LV108 and LV102 and cloned in the pGEM-T vector, which resulted in pABV456. The wild-type and mutated ORF7 genes were excised from pABV431, pABV453, pABV455, and pABV463 by digestion with *Pac*I (blunt ended) and *Hpa*I and from pABV456 by digestion with *Hpa*I and *Sma*I. These genes were subsequently inserted in the dephosphorylated *Sma*I site of Semliki Forest virus expression vector pSFV1 (Liljeström and Garoff, 1991). Plasmids pABV470, pABV460, pABV462, pABV518, and pABV471 containing the respective ORF7 genes in the correct orientation were further tested for expression of the N protein.

In vitro transcription and transfection of Semliki Forest virus ORF7 RNA

The pSFV1 plasmids containing different ORF7 constructs were linearized by digestion with *Spe*I and transcribed *in vitro*, according to the protocol of Liljeström and Garoff (1993). The synthesized RNA was transfected to BHK-21 cells in 15-mm wells of 24-well plates with lipofectin as described by Liljeström and Garoff (1993). At 24 h after transfection, cells were fixed with ice-cold 4% (v/v) paraformaldehyde, and the N protein expressed by the various ORF7 constructs was stained with mAbs in the IPMA, essentially as described by Wensvoort *et al.* (1986).

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